

RESEARCH PAPER

GPR55 promotes migration and adhesion of colon cancer cells indicating a role in metastasis

J Kargl¹, L Andersen¹, C Hasenöhr¹, D Feuersinger¹, A Stančić¹, A Fauland², C Magnes², A El-Heliebi^{3,4}, S Lax⁵, S Uranitsch⁶, J Haybaeck⁷, A Heinemann¹ and R Schicho¹

¹Institute of Experimental and Clinical Pharmacology, Medical University of Graz, Graz, Austria, ²HEALTH – Institute for Biomedicine and Health Sciences, Joanneum Research Forschungsgesellschaft m.b.H., Graz, Austria, ³Institute of Cell Biology, Histology and Embryology, Medical University of Graz, Graz, Austria, ⁴Biobank Graz, Medical University of Graz, Graz, Austria, ⁵Department of Pathology, General Hospital Graz West, Graz, Austria, ⁶Department of Surgery, St John of God Hospital Graz, Graz, Austria, and ⁷Institute of Pathology, Medical University of Graz, Graz, Austria

Correspondence

Rudolf Schicho and Julia Kargl, Institute of Experimental and Clinical Pharmacology, Medical University of Graz, Universitätsplatz 4, 8010 Graz, Austria.

E-mail: rudolf.schicho@medunigraz.at; julia.kargl@medunigraz.at

Received

24 September 2014

Revised

31 August 2015

Accepted

24 September 2015

BACKGROUND AND PURPOSE

Tumour cell migration and adhesion constitute essential features of metastasis. G-protein coupled receptor 55 (GPR55), a lysophospholipid receptor, has been shown to play an important role in carcinogenesis. Here, we investigated the involvement of GPR55 in migration and metastasis of colon cancer cells.

EXPERIMENTAL APPROACH

Adhesion and migration assays using the highly metastatic colon cancer cell line HCT116 and an *in vivo* assay of liver metastasis were performed. The GPR55 antagonist CID16020046, cannabidiol, a putative GPR55 antagonist and GPR55 siRNA were used to block GPR55 activity in HCT116 colon cancer cells.

KEY RESULTS

HCT116 cells showed a significant decrease in adhesion to endothelial cells and in migration after blockade with CID16020046 or cannabidiol. The inhibitory effects of CID16020046 or cannabidiol were averted by GPR55 siRNA knock down in cancer cells. The integrity of endothelial cell monolayers was increased after pretreatment of HCT116 cells with the antagonists or after GPR55 siRNA knockdown while pretreatment with lysophosphatidylinositol (LPI), the endogenous ligand of GPR55, decreased integrity of the monolayers. LPI also induced migration in GPR55 overexpressing HCT116 cells that was blocked by GPR55 antagonists. In a mouse model of metastasis, the arrest of HCT116 cancer cells in the liver was reduced after treatment with CID16020046 or cannabidiol. Increased levels of LPI (18:0) were found in colon cancer patients when compared with healthy individuals.

CONCLUSIONS AND IMPLICATIONS

GPR55 is involved in the migratory behaviour of colon carcinoma cells and may serve as a pharmacological target for the prevention of metastasis.

Abbreviations

CBD, cannabidiol; CMV, cytomegalovirus; GPR55, G-protein coupled receptor 55; LPA, lysophosphatidic acid; LPI, lysophosphatidylinositol; MEK, mitogen-activated protein kinase kinase; NFAT, nuclear factor of activated T-cells; ROCK, Rho-associated coiled-coil containing protein kinase 1

Tables of Links

TARGETS			LIGANDS
GPCRs^a	Nuclear hormone receptors^c	Enzymes^d	Cannabidiol
5-HT _{1A} receptor	PPAR γ	ERK1	CID16020046
GPR55		ERK2	Lysophosphatidic acid (LPA)
LPA receptors		MEK1	Lysophosphatidylinositol (LPI)
Ion channels^b		MEK2	Y27632
TRPV2		ROCK	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (^{a,b,c,d}Alexander *et al.*, 2013a,b,c,d).

Introduction

The G-protein coupled receptor 55 (GPR55), a lysophosphatidylinositol (LPI) receptor, has recently emerged as a potential novel target in antitumour treatment because of its involvement in several hallmarks of cancer, such as proliferation, invasion and angiogenesis (Henstridge *et al.*, 2011; Ross, 2011). GPR55 was originally described as a receptor responsive to natural, synthetic and endogenous cannabinoids like anandamide (Ryberg *et al.*, 2007), however, the only consistent data eventually pointed to LPI as its true endogenous ligand (Henstridge *et al.*, 2009; Oka *et al.*, 2009). High levels of GPR55 have been detected in the CNS, spleen and the gastrointestinal tract (Henstridge *et al.*, 2011). It has been also identified in several types of cancer cells, such as glioma, melanoma, breast and pancreatic cancer cells (Ford *et al.*, 2010; Andradas *et al.*, 2011; Piñeiro *et al.*, 2011; Pérez-Gómez *et al.*, 2012). Unlike cannabinoid receptors, GPR55 signals through G $\alpha_{12/13}$ and G α_q proteins initiating excitatory rather than inhibitory effects (Lauckner *et al.*, 2008). In line with this concept, GPR55 has been implicated in the development of neuropathic and inflammatory pain through modulation of pro-inflammatory cytokines (Staton *et al.*, 2008). Structurally, GPR55 is related to several cancer-relevant GPCRs including GPR35, GPR92 and GPR23 (Fredriksson *et al.*, 2003). Because signalling pathways initiated by G $\alpha_{12/13}$ subunits contribute to cancer-related mechanisms such as proliferation, migration and invasion (Juneja and Casey, 2009), a role for GPR55 in the development of cancer has been suspected. Indeed, overexpression of GPR55 enhances whereas silencing of GPR55 reduces viability of breast, glioma and pancreatic cancer cells (Andradas *et al.*, 2011). The receptor is up-regulated in tumours of human pancreatic and skin cancers and positively correlates with tumour aggressiveness in skin and oral squamous cell carcinomas and glioblastoma (Andradas *et al.*, 2011; Pérez-Gómez *et al.*, 2012). A recent study using a model of skin cancer in GPR55 knock-out mice revealed that proliferation of epidermal cells was reduced (Pérez-Gómez *et al.*, 2012). It is most likely that GPR55-mediated proliferation of cancer cells involves MAPK pathways (Andradas *et al.*, 2011; Piñeiro *et al.*, 2011). Contrary to these studies, antiproliferative effects of GPR55 have been described in cholangiocarcinoma cells (Huang *et al.*, 2011).

Little is known about the role of GPR55 in the metastatic behaviour of cancer cells, and colon cancer in particular. GPCRs play a central role in metastasis owing to their ability to regulate cell migration (Dorsam and Gutkind, 2007). Also, Ca²⁺ homeostasis is altered in metastatic cancer cells (Prevarskaya *et al.*, 2011). Previous studies indicate a role for GPR55 in the migration of endothelial, skin and breast cancer cells (Ford *et al.*, 2010; Zhang *et al.*, 2010; Pérez-Gómez *et al.*, 2012). These studies and other findings on the cellular pathways initiated by LPI point to a possible role for the GPR55-LPI axis in colon cancer cell migration and invasion. Activation of GPR55 by LPI initiates downstream events crucial for the migration of cancer cells, such as Ca²⁺ mobilization (Henstridge *et al.*, 2009), ERK1/2 phosphorylation (Andradas *et al.*, 2011; Piñeiro *et al.*, 2011) and activation of serum response element (Kargl *et al.*, 2012). In addition, LPI also activates migration-promoting signalling protein RhoA in GPR55-HEK293 cells (Henstridge *et al.*, 2009) and human neutrophils (Balenga *et al.*, 2011).

To investigate the role of GPR55 and LPI in the migration and possible metastasis of colon cancer cells, we used the highly metastatic colon cancer cell line HCT116 (Rajput *et al.*, 2008) to demonstrate the involvement of GPR55 in colon cancer. For pharmacological blockade, we used the selective GPR55 antagonist CID16020046 (Kargl *et al.*, 2013a) and cannabidiol, which may act as a GPR55 inhibitor (Ryberg *et al.*, 2007; Anavi-Goffer *et al.*, 2012). Because cannabidiol has been shown to use multiple mechanisms for prevention of experimental carcinogenesis (Aviello *et al.*, 2012), we were interested whether cannabidiol would exert its anticarcinogenic effects in our experiments via GPR55. Our *in vitro* assays demonstrated that GPR55 is involved in adhesion and migration of colon cancer cells. Using an *in vivo* model of tumour cell metastasis, we show that after intrasplenic injection of HCT116-CMVp-Luc colon cancer cells, the arrest of cells is reduced in liver tissue of mice treated with CID16020046 or cannabidiol. We also detected increased LPI (18:0) content in the blood of colon cancer patients when compared with healthy donors. This study provides evidence that GPR55 is involved in the metastatic behaviour of colon cancer cells.

Methods

Cell culture and drugs

Colon cancer cells (HCT116, HT-29 and SW480) were purchased from Interlab Cell Line Collection, Genoa, Italy; HCT-CMVp-Luc cells were kindly provided by Dr Antje Siegert, EPO, Berlin, Germany. Overexpression of human 3xHA-GPR55 or vector alone (pcDNA3.1) in HCT116 cells was performed as previously described using Lipofectamine 2000 (Kargl *et al.*, 2012) and verified by quantitative real time PCR (qPCR). HCT116 and HCT116-CMVp-Luc were cultured in McCoy's 5A medium (PAA Laboratories, Pasching, Austria) supplemented with 10% FBS and 1% PenStrep (PAA). Cells were grown at 37°C in 5% CO₂-humidified atmosphere. No passage higher than seven was used for experiments. Before all assays, cells were starved for 4–24 h in Opti-MEM (Life Technologies, Invitrogen, Vienna, Austria) supplemented with 1 or 0.5% FBS. HUVECs (Lonza, Verviers, Belgium) were cultured on 1% gelatine-coated cell culture dishes in endothelial basal medium supplemented with endothelial growth medium (Lonza). Cannabidiol was obtained from Tocris Bioscience (Bristol, UK) and diluted in DMSO. The GPR55 antagonist CID16020046 ((4-[4-(3-hydroxyphenyl)-3-(4-methylphenyl)-6-oxo-1 H,4 H,5 H,6 H-pyrrolo [3,4-c] pyrazol-5-yl] benzoic acid) was purchased from Molport (Riga, Latvia) and dissolved in DMSO. LPI (Sigma, St Louis, MO, USA) was dissolved in deionized sterile water.

PCR

RNA isolation, reverse transcription PCR (RT-PCR) and qPCR were performed as previously described (Kargl *et al.*, 2013b) using the following primers for GPR55: (forward: 5'-CCT CCCATTCAAGATGGTCC-3'; reverse: 5'-GACGCTTCCGTA CATGCTGA-3') and GAPDH: (forward: 5'-ATGGGGAAGGTG AAGGTCG-3'; reverse: 5'-GGGGTCATTGATG-GCAACAATA-3'). Data from real-time PCR were analysed using the $2^{-\Delta\Delta Ct}$ method or calculated by using a standard curve. A pcDNA3.1 plasmid encoding the GPR55 gene served for standard curve calculation, and the absolute mRNA copy number was assessed.

Adhesion assay

HUVEC cells were seeded in black wall clear bottom 96-well plates and allowed to adhere for 48 h before each experiment. HUVEC cell monolayers were then starved in EBM incomplete media +1% FBS for 1 h and subsequently treated with TNF α (10 ng·mL⁻¹) for 4 h. HCT116 cells or HCT116 cells transfected with siRNA were starved in EBM incomplete +1% FBS for 1 h and then treated with CID16020046, cannabidiol or with specific inhibitors targeting mitogen-activated protein kinase kinase (MEK)1/2 (PD184161; Cayman) and Rho-associated coiled-coil containing protein kinase 1 (ROCK) (Y27632 [Sigma Aldrich, St Louis, MO, USA] and H-1152 [Tocris]) for 1 h at the indicated concentrations. Cells were washed once and loaded with calcein-AM (1:1000) in EBM incomplete +1% FBS for 15 min at 37°C in the dark. After two additional washing steps, HCT116 cells were added to the HUVEC monolayer and incubated for 30 min at 37°C at 90 r.p.m. shaking. Cells were washed twice with HBSS before signals of adherent HCT116 cells were measured in

a FLEX station II. Data are expressed as relative fluorescence units (RFU).

Cell viability assay

The number of viable cells was determined using the Cell Titer 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) as previously described (Kargl *et al.*, 2013b).

F-actin staining

HCT116 cells were grown on poly-D-lysine-coated slides and starved for 4 h. Cells were pretreated with DMSO, 1 μ M cannabidiol or CID16020046 for 10 min and stimulated with 1 μ M LPI for 15 min. Subsequently, cells were fixed, permeabilized, blocked and incubated with the Texas Red-conjugated phalloidin. Cells were wet-mounted onto microscopy slides with DAPI mounting medium (Vectashield®; Vector Laboratories, Inc., Burlingame, CA, USA).

Spot assays

The assay was carried out according to Wiggins and Rappoport (2010) with minor modifications. Briefly, sterile agarose was dissolved in sterile water to a final concentration of 0.5% and supplemented with vehicle, 10% FBS or 10% FBS + 2.5 μ M LPI. Ten microlitre spots of agarose solution were pipetted rapidly on a 24-well plate and left to dry at room temperature for at least 2 h. HCT116 cells were detached, washed in PBS and 5×10^5 cells per well were seeded in Opti-MEM + 1% FBS per well in the spot-containing 24-well plate, where they were allowed to adhere for at least 2 h at 37°C. Subsequently, cells were treated with vehicle or compound and allowed to migrate into the spot for 17 h. For spot analysis, four pictures per spot were taken under a 10 \times objective of an Olympus IX 70 microscope. The migration index was calculated as treatment per control (control set at 1).

Transwell migration assay

Migration assays were performed in 24-well Transwell plates with 8 μ m membrane inserts (Corning Inc., Lowell, MA, USA). Cells were starved in Opti-MEM and then incubated with GPR55 inhibitor CID16020046 (1, 2.5 and 5 μ M), cannabidiol (1 and 2.5 μ M) or with MEK1/2 inhibitor PD184161 and ROCK inhibitors Y27632 and H-1152 for 1 h. Thereafter, a suspension of 1×10^5 cells was placed in the upper well; as a chemoattractant, 1% FBS in Opti-MEM \pm 1 μ M LPI was added into the bottom well. Cells were allowed to migrate for 17 h at 37°C and 5% CO₂ in a humidified incubator. Upon completion of migration, the upper sides of the filters were cleaned with a cotton swab, and filters were dried and fixed in formaldehyde for 30 min. After a wash in PBS, filters were mounted and coverslipped in Vectashield®. Cell nuclei were counted under a fluorescent microscope (Olympus IX 70). Each migration experiment was performed in duplicates. The average number of migrated cells was determined from at least five independent experiments.

ECIS assay

Electric cell-substrate impedance sensing (ECIS®, Applied Biophysics, Troy, NY, USA) with gold electrode arrays of the

type 8W10E+ was used to monitor the integrity of HUVEC cell monolayers at real-time *in vitro*. ECIS array wells were activated with L-cysteine (10 mM) and precoated with 1% gelatine for 30 min at 37°C. Then, wells were allowed to equilibrate with 200 µL HUVEC medium for 15 min at 37°C before HUVEC cells were added in additional 200 µL HUVEC medium to each well (1.5×10^5 cells per well). Monolayer formation was followed by impedance measurements. HUVEC cells were starved and challenged with monodisperse cell suspensions of HCT116 cells in fresh HUVEC starvation medium at a ratio of 1:1 (colon carcinoma vs. endothelial cells). Cancer cells were incubated with the appropriate concentrations of compounds in HUVEC starvation medium for 1 h before adding them to the well. The impedance of the challenged endothelial cell layer was monitored for 24 h.

Tracking of tumour cells in the liver of C57BL/6 mice

All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). Experimental procedures in mice were approved by the Austrian Federal Ministry of Science and Research (protocol number 66.010/0090–II/3b/2012) and performed in accordance with national and international guidelines. Procedures were performed as humanely as possible to minimize all suffering. C57BL/6 (males, 5–9 weeks old, 20–26 g) were obtained from Charles River (Sulzfeld, Germany) and kept in house for 2 weeks before the experiments. Mice were housed in plastic sawdust floor cages at constant temperature (22°C) and a 12:12 h light–dark cycle with free access to standard laboratory chow and water. In total, 55 mice were used for the experiments. Tumour cell arrest in the liver was detected and quantified using a luciferase assay. HCT116 cells expressing a firefly luciferase (Luc-2) under the control of a cytomegalovirus (CMV) promoter (HCT116-CMVp-Luc) (Sack *et al.*, 2011) were injected into the spleen at 1×10^6 cells in 50 µL volume under ketamine (100 mg·kg^{−1}) and xylazine (10 mg·kg^{−1}) anaesthesia. Heart rate was monitored during anaesthesia using the *Physio Suite* non-invasive monitoring system (Kent Scientific, Torrington, CT, USA). Three and a half hours after the injection, the left lobe of the liver was removed, rinsed in PBS, blotted and weighed and quickly transferred into lysis buffer [25 mM TRISphosphate (pH 7.8), 10% glycerol, 1% Triton-X-100, 1 mg·mL^{−1} BSA, 2 mM EGTA and 2 mM DTT]. After sonication and centrifugation, 100 µL of supernatant was added to assay reagent (reaction buffer, 1 mM luciferin, 2 mM ATP). Reaction buffer consisted of 25 mM glycylglycine, 15 mM MgSO₄, 4 mM EDTA, 15 mM K₂PO₄ (pH 7.8), 1 mM DTT and 1 mM CoA. After 1 min, luminescence was measured for 5 s at 562 nm at a TopCounter (Top Count NXT; Packard Instrument Company, Meriden, CT, USA). Luminescence values were normalized to liver wt and expressed as relative light units.

Human blood samples

Blood samples were provided as part of the *Oncotrack* project (<http://www.oncotrack.eu/>) by the General Hospital Graz West, St John of God Hospital Graz, Graz, Austria, and by the Institute of Experimental and Clinical Pharmacology, Medical University of Graz, Austria. Blood was collected from colon cancer patients and healthy individuals ($n = 7$), drawn

into heparin-containing plasma separation tubes (Greiner-Bio-One, Austria) and centrifuged within 2 h at 1600 × *g* for 10 min. Plasma was then transferred into cryotubes without disturbing the buffy coat layer. The specimens were stored at −80°C until use. Written informed consent was obtained from all patients. Ethical approval was granted by the ethics committee of the Medical University of Graz and confirmed by the ethics committee of the St John of God Hospital Graz (23-015 ex 10/11 and 17-291 ex 05/06).

LC-MS of LPI

Lipid extraction was carried out from 150 µL of sample in the presence of 10 µL of LPI 17:1 (100 µM) as internal standard according to Matyash *et al.* (2008). LC-MS measurements for lipid quantification were performed with slight modifications as previously described in Fauland *et al.* (2011). Briefly, chromatographic separation was performed on a Kinetex reversed-phase C18 2.1 × 150 mm (2.6 µm) column (Phenomenex, Torrance, USA). A LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was used. The instrument was operated in preview mode for parallel MS/MS spectra in the linear ion trap, while running the Orbitrap in full scan mode at 100 000 resolution (*m/z* 400) from *m/z* 490 to 1100 in negative ESI-mode. Helium was used as the gas for linear ion trap collision-induced dissociation spectra. From the LTQ-FT preview scan, the three most abundant ions were selected in data-dependent acquisition, fragmented in the linear ion trap analyser and ejected at nominal mass resolution.

Statistical analysis

Data are presented as means ± SEM of at least $n = 5$ observations, unless stated otherwise. Statistical analyses were performed using one-way ANOVA followed by a Bonferroni's or Tukey's *post hoc* test and Student's *t*-test run on GraphPad Prism® software. *P*-values of <0.05 were considered significant.

Results

GPR55 contributes to adhesion of HCT116 colon cancer cells onto HUVEC cell monolayers. We first evaluated GPR55 expression in several different colon cancer cell lines by qPCR with highest expression observed in HCT116 cells (Figure S1). HCT116 cells were pretreated for 1 h with either 1 or 2.5 µM (Figure 1A) of GPR55 antagonist CID16020046 (referred as CID in graphs) or cannabidiol respectively. The two compounds significantly diminished adhesion of HCT116 cells to a HUVEC cell monolayer at both concentrations used. At 2.5 µM of CID16020046, adhesion was diminished by more than 50%. Similar effects were observed using GPR55 siRNA (Figure 1B). The inhibitory effect of CID16020046 and cannabidiol on HCT116 cancer cell adhesion was absent when GPR55 expression was silenced with siRNA indicating a direct effect of CID16020046 and cannabidiol on GPR55 (Figure 1C). Knockdown of GPR55 in HCT116 cells, therefore, corroborated the results seen after the pharmacological inhibition of GPR55 activity. The siRNA knockdown of GPR55 resulted in a 50% loss of GPR55 mRNA levels (Figure 1D).

GPR55 is involved in the migration of HCT116 colon cancer cells. We then investigated the role of GPR55 in the

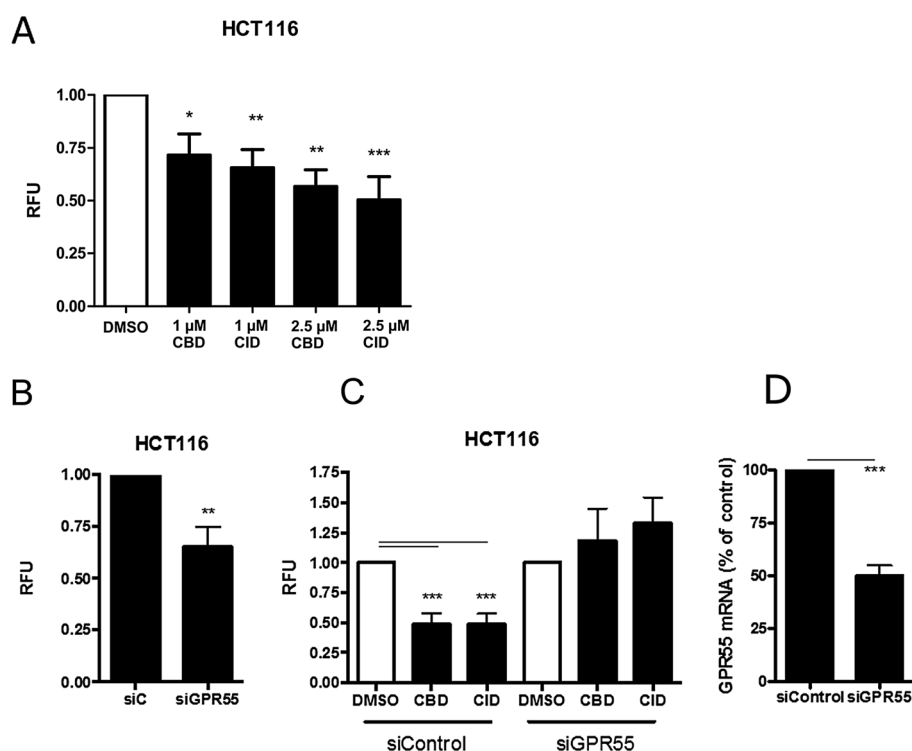


Figure 1

GPR55 contributes to the adhesion of HCT116 cells onto a HUVEC cell monolayer. Adhesion assays were performed with HCT116 cells. The cells were either pretreated with GPR55 antagonist CID16020046 (CID), cannabidiol (CBD) or vehicle (DMSO). (A) Treatment with 1 μ M CID or cannabidiol and with 2.5 μ M CID or cannabidiol significantly diminished adhesion of HCT116 cells. (B) Knockdown of GPR55 expression with siRNA (siGPR55) also diminished adhesion of HCT116 cells. (C) Cannabidiol and CID (1 μ M) were not effective in diminishing adhesion of HCT116 cells already knocked down with siRNA against GPR55 (siGPR55) as compared with cells transfected with control siRNA (siControl). (D) GPR55 knockdown caused 50% reduction in GPR55 mRNA levels. To knock down GPR55 expression, siRNA targeting GPR55 (ON-TARGETplus siRNA; Dharmacon, Thermo Fisher Scientific Inc., Waltham, MA, USA) and control siRNA (Microsynth, Balgach, Switzerland) were transfected using Lipofectamine RNAiMAX following the manufacturer's instructions (Life Technologies). The extent of the siRNA knockdown in HCT116 cells was verified for each experiment by qPCR of GPR55 mRNA. $n = 6$; ANOVA with Bonferroni's *post hoc* analysis or Student's *t*-test; ** $P < 0.01$; *** $P < 0.001$. RFU, relative fluorescence units.

migration of HCT116 cells towards a chemoattractant using agarose spot and Transwell migration assays (Figures 2 and 3). Ten percent FBS and 10% FBS + 2.5 μ M LPI (agarose spot assay, Figure 2) and 1% FBS and 1% FBS + 1 μ M LPI (Transwell migration assay, Figure 3) were used as chemotactic factors for HCT116 cells. Preincubation of HCT116 cells with 1 μ M of CID16020046 or cannabidiol significantly reduced migration in both experimental settings (Figures 2A, B and 3A, B). GPR55 knockdown significantly decreased GPR55-mediated HCT116 cell migration (Figure 2C). The inhibiting effect of CID16020046 and cannabidiol on HCT116 cancer cell migration was averted in cells following GPR55 knockdown, indicating a direct effect of CID16020046 and cannabidiol on GPR55 (Figure 2D). In line with another report (Ford *et al.*, 2010), LPI alone failed to significantly act as a chemoattractant for naïve HCT116 cells in the Transwell migration assay (Figure 3E); however, it increased migration in GPR55 overexpressing HCT116 cells (Figure 3F). Incubation with 2.5 μ M CID16020046 reversed the increase indicating that the inhibitor blocked LPI induced responses (Figure 3G). After incubation with 2.5 μ M cannabidiol, the LPI-induced migratory response in GPR55 overexpressing HCT116 cells was decreased by around 50% (Figure 3H). LPI-induced formation of F-actin filaments (arrows in Figure 3I) while

preincubation with CID16020046 and cannabidiol inhibited that effect (Figure 3I). Representative pictures of migrated HCT116 cells from the spot and Transwell assay are shown in Figures 2E and 3J respectively. Preincubation with CID16020046 or cannabidiol over the length of the assays did not decrease cell viability (Figure S2).

Blockade of GPR55 in HCT116 colon cancer cells changes the integrity of HUVEC cell monolayers. To study whether GPR55 could play a role in the passage of cancer cells through an endothelial barrier, another hallmark of cancer cell metastasis, we performed impedance-based assays (ECIS®) to examine whether co-culture with HCT116 cells can decrease the integrity of a HUVEC cell monolayer whereby a decrease in resistance correlates with a decrease in the monolayer's integrity (Figure 4). Co-culture of HCT116 cells with the HUVEC monolayer decreased resistance (Figure 4A), while pretreatment of HCT116 cells with 1 μ M of CID16020046 or cannabidiol inhibited this decrease (seen as an increase of resistance compared with the DMSO control, Figure 4A). Similar effects were observed with GPR55 knockdown cells (Figure 4B), while a loss of the monolayer integrity (seen as a decrease in resistance compared with untreated in

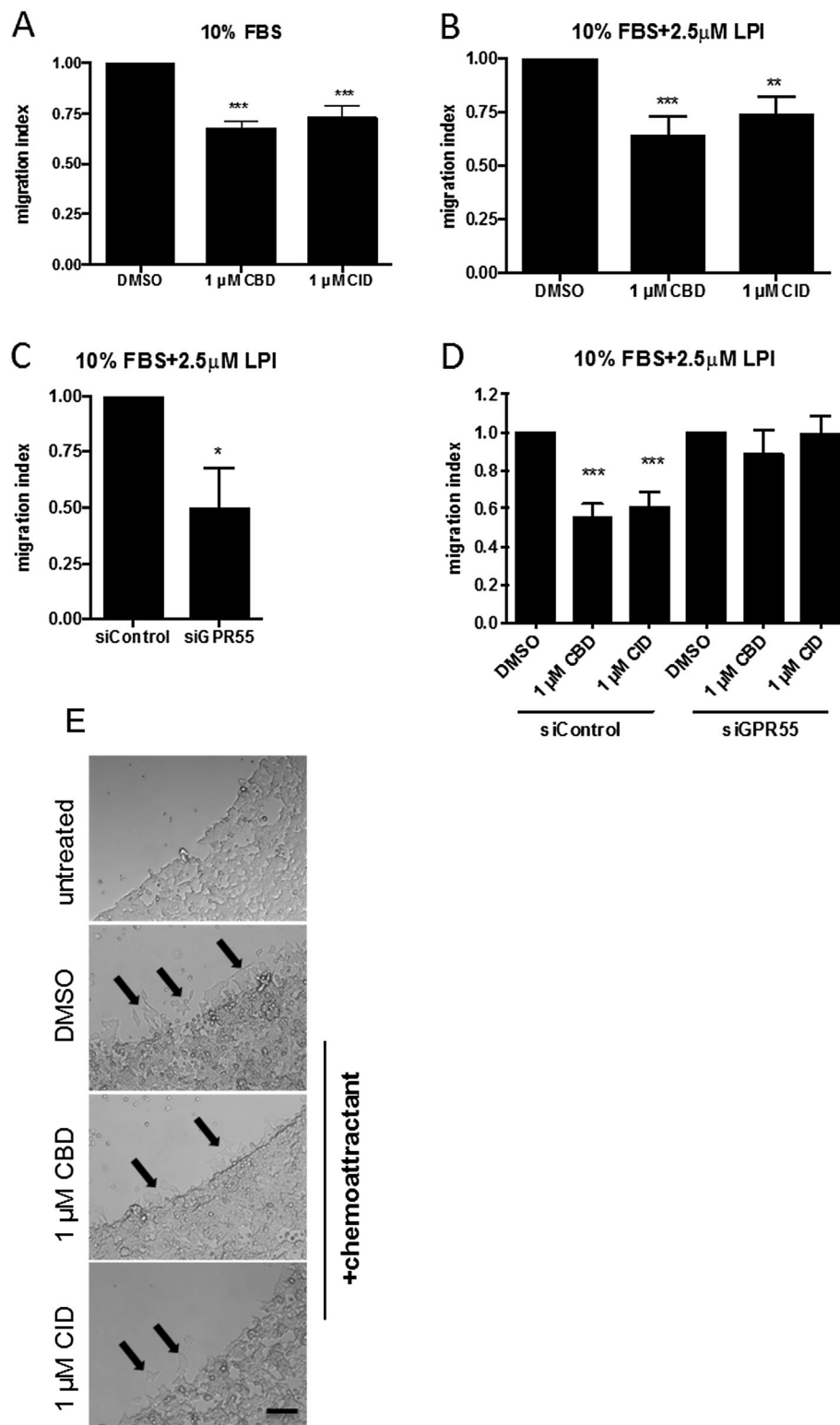


Figure 2

GPR55 is involved in the invasion of HCT116 colon cancer cells. To study chemotactic invasion, agarose spot assays were performed using (A) 10% FBS and (B) 10% FBS + 2.5 μ M LPI as chemoattractants. Treatment with 1 μ M CID16020046 (CID) or cannabidiol (CBD) reduced the invasion of HCT116 cells in comparison with vehicle treatment (DMSO). (C) Knockdown of GPR55 expression with siRNA (siGPR55) also reduced the invasion of HCT116 cells into the agarose spot. (D) Cannabidiol and CID (1 μ M) were not effective in diminishing the invasion of HCT116 cells already knocked down with siRNA against GPR55 (siGPR55) as compared with cells transfected with control siRNA (siControl). (E) Representative images of HCT116 cell invasion (arrows) into the agarose spot. Size bar: 200 μ m. $n = 3$; ANOVA with Bonferroni's *post hoc* test or Student's *t*-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

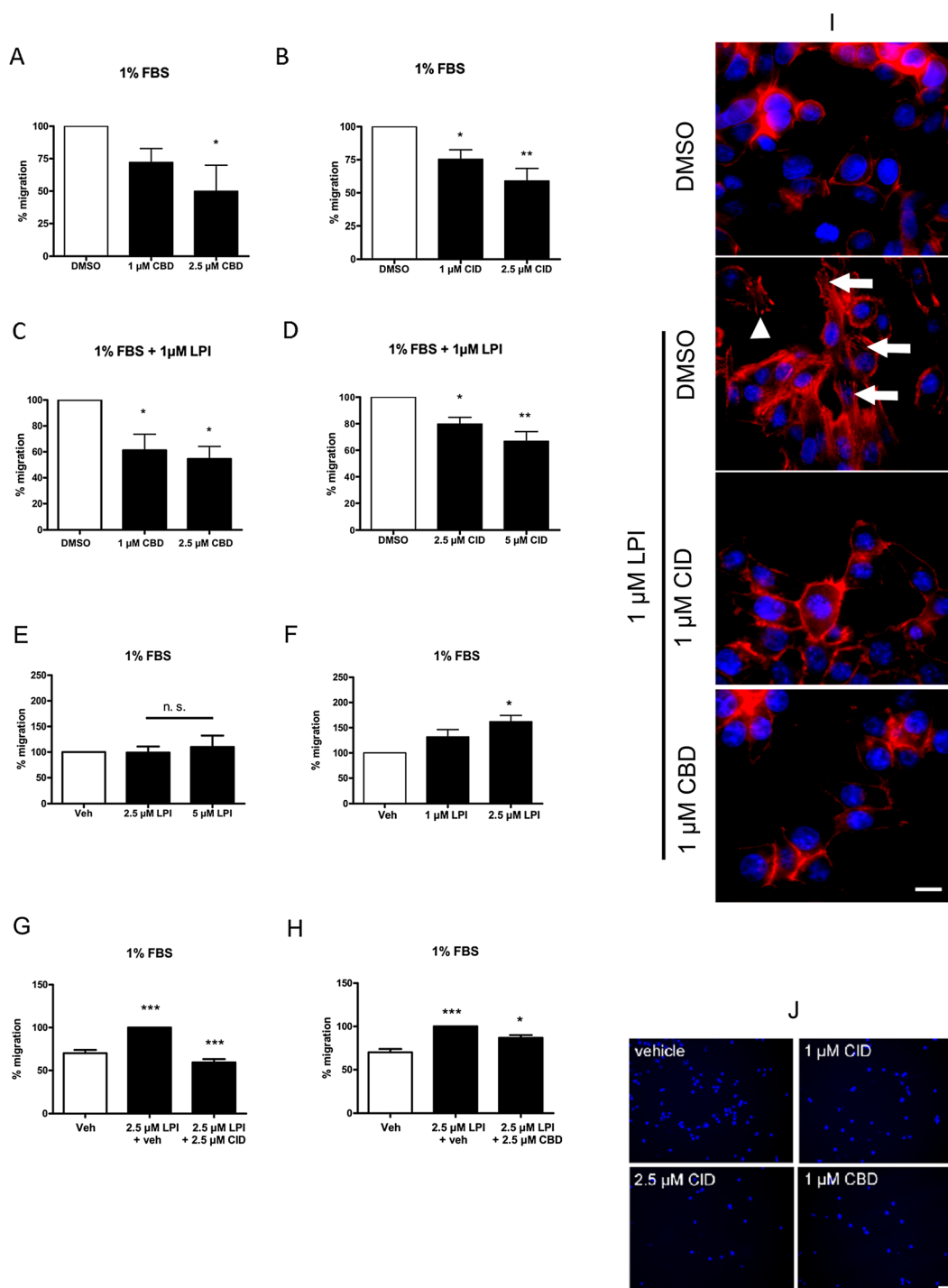


Figure 3

GPR55 is involved in the migration of naïve and GPR55 overexpressing HCT116 colon cancer cells. Transwell migration assays were employed to study the role of GPR55 in the migration of naïve and GPR55 overexpressing HCT116 colon cancer cells. (A, B) Both cannabidiol (CBD) and CID16020046 (CID) at 1 and 2.5 μ M were effective in inhibiting migration of naïve HCT116 cells using 1% FBS as a chemoattractant. $n = 5-6$; ANOVA; Tukey's *post hoc* test. (C, D) Inhibition of migration was similarly effective when using 1% FBS and 1 μ M LPI as chemoattractant. $n = 5-6$; ANOVA; Tukey's *post hoc* test. (E) LPI failed to induce migration in naïve HCT116 cells, but it induced migration in GPR55 overexpressing HCT116 cells (F) (veh, vehicle). (G, H) Addition of 2.5 μ M CID16020046 (CID) or 2.5 μ M cannabidiol decreased the LPI-induced responses in GPR55 overexpressing HCT116 cells (veh, vehicle [DMSO]). $n = 5-10$; ANOVA; Tukey's *post hoc* test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (I) LPI (1 μ M) induced formation of F-actin filaments (arrows) and focal adhesion plaques (arrowhead) in naïve HCT116 cells incubated with solvent (DMSO), while pre-incubation with 1 μ M CID and 1 μ M cannabidiol inhibits that effect. Nuclei were stained with DAPI. Size bar: 20 μ m. (J) Representative images from cells that have migrated to the lower side of the migration filter in the Transwell assay. For visualization and counting, cells were stained with DAPI. Size bar: 200 μ m.

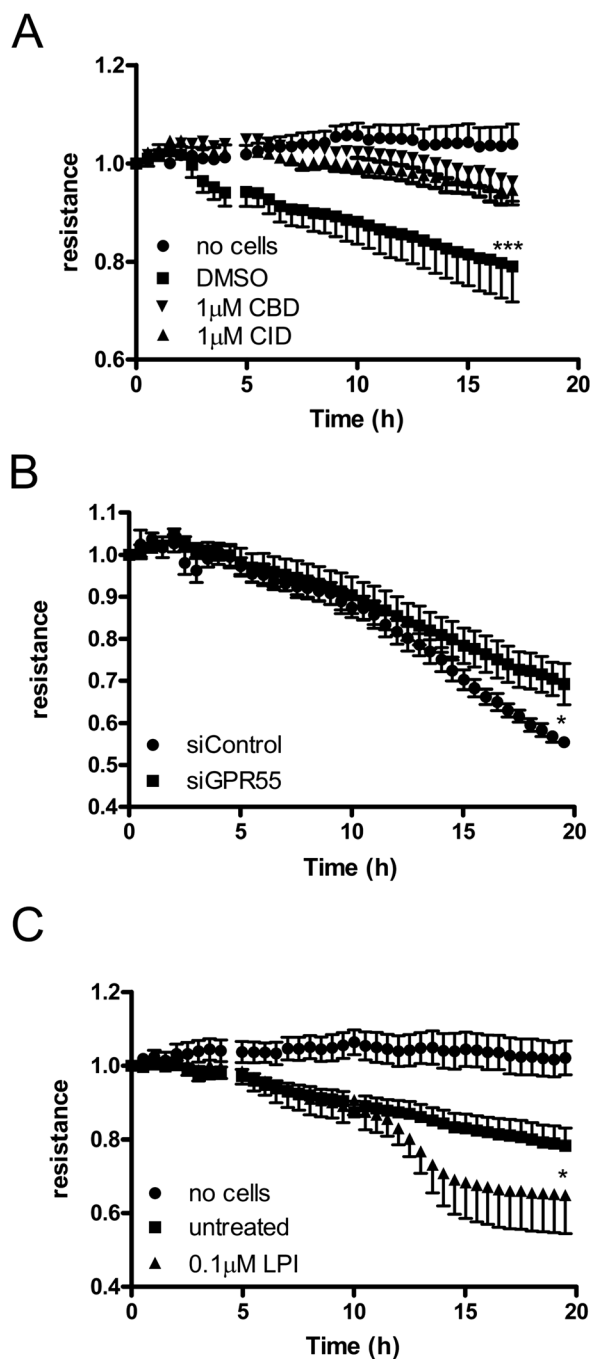


Figure 4

Pretreatment of HCT116 colon cancer cells with GPR55 inhibitors alters the integrity of an endothelial cell monolayer. An impedance-based assay (ECIS®) of HCT116 cells co-incubated with a HUVEC cell monolayer is shown. (A) HCT116 cells pretreated with vehicle only (DMSO) strongly reduced resistance indicative of a decrease in the integrity of the HUVEC cell monolayer, while pretreatment of HCT116 cells with 1 μ M CID16020046 (CID) or cannabidiol (CBD) restored resistance. (B) Resistance was also preserved when GPR55 was knocked down with siRNA in HCT116 cells (siGPR55) as compared with cells transfected with control siRNA only (siControl). (C) Pretreatment of HCT116 cells with LPI (0.1 μ M) reduced resistance indicating enhanced disruption of the HUVEC cell monolayer; ANOVA with Bonferroni's *post hoc* test. $n = 3-5$; * $P < 0.05$; *** $P < 0.001$.

Figure 4C) was observed after pretreatment of HCT116 cells with GPR55 ligand LPI (0.1 μ M).

HCT116 cancer cell adhesion and migration are ERK1/2 dependent. We used inhibitors of ERK1/2 and ROCK to investigate possible signalling pathways that participate in adhesion and migration of HCT116 cells. These two signalling molecules have been implicated in GPR55-mediated tumour growth (Andradas *et al.*, 2011) and migration of neutrophils (Balenga *et al.*, 2011).

The potent MEK1/2 inhibitor PD184161, which previously showed total inhibition of bicuculline-induced ERK1/2 phosphorylation at 5 μ M in neuronal cell cultures (Gladbach *et al.*, 2014), blocked the ability of HCT116 cells to adhere by more than 50% (Figure 5A), while Y27632, a selective inhibitor of the Rho/ROCK pathway, failed to inhibit adhesion in naïve HCT116 cells (Figure 5A). In agarose spot (Figure 5B) and Transwell migration assays (Figure 5C), similar observations were made. Higher concentrations of PD184161 than 5 μ M significantly reduced cell viability (Figure S3) and were, therefore, not used in our experiments. Only in adhesion assays, which were evaluated after 1 h, 10 μ M of PD184161 were used. No decrease in cell viability was detected at that time point (data not shown). Inhibition of the Rho/ROCK pathway using Y27632 significantly increased migration and adhesion of naïve HCT116 cells (Figure 5B and C).

In GPR55 overexpressing HCT116 cells, 5 μ M of PD184161 blocked the LPI-induced migratory response in the Transwell migration assay (Figure 5D). Similar to ROCK inhibitor Y2763, a different ROCK inhibitor, H-1152, was unable to significantly decrease the LPI-induced migratory response in the Transwell assay at 10 nM (Figure S3A) and had no effect in the adhesion assay using naïve HCT116 cells (Figure S3B).

GPR55 is involved in the arrest of HCT116 colon cancer cells in the liver after intrasplenic inoculation. To examine whether GPR55 might also play an important role in migration and invasion, we used a model of liver metastasis and injected HCT116 cells that expressed a firefly luciferase (Luc-2) under the control of a CMV promoter (HCT116-CMVp-Luc) (Sack *et al.*, 2011) intrasplenically into mice. GPR55 expression was detected in HCT116-CMVp-Luc cells comparable with HCT116 cells (Figure S4). In order to quantify arrested cancer cells, liver tissue was evaluated for luciferase activity 3.5 h after injection of cells (1×10^6 cells in 50 μ L; Sack *et al.*, 2011). To visually demonstrate the actual arrest of HCT116-CMVp-Luc cells in the liver, cells were transfected with GFP and visualized by fluorescence microscopy in liver sections 3.5 h after intrasplenic inoculation (Figure 6D). No cells with a passage number higher than seven were used for these experiments. *In vivo* treatment (i.p.) with 10 mg·kg⁻¹ CID16020046 or with 5 mg·kg⁻¹ cannabidiol led to a significant reduction in arrest of intrasplenically injected HCT116-CMVp-Luc cells, as evaluated by luciferase activity in the liver (Figure 6A and B). Preincubation of HCT116-CMVp-Luc cells only with CID16020046 (1 and 2.5 μ M) 1 h prior to their intrasplenic injection concentration-dependently inhibited luciferase activity in the liver (Figure 6C).

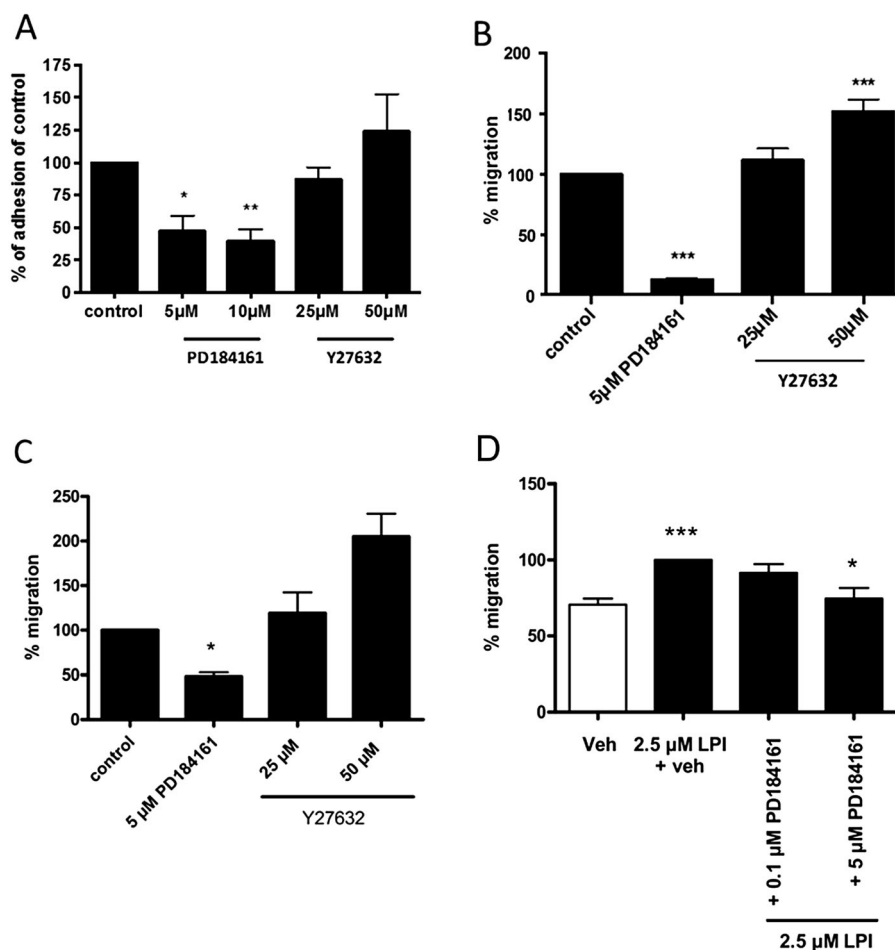


Figure 5

Adhesion and migration of HCT116 cancer cells are ERK1/2 dependent. Adhesion (A) and migration assays (B–D) were performed using naïve and GPR55 overexpressing HCT116 cells. Naïve HCT116 cells were either pretreated with ERK1/2 inhibitor PD184161 (5 μ M) or ROCK inhibitor Y27632 (25 and 50 μ M) for 1 h and (A) adhesion ($n = 5–6$), (B) agarose spot ($n = 3$) and (C) Transwell migration assays ($n = 6–8$) were performed. PD184161 significantly diminished HCT116 cell adhesion to HUVEC cell monolayer and significantly reduced cell migration, while Y27632 increased the effects. (D) In GPR55 overexpressing HCT116 cells, 5 μ M of PD184161 blocked the LPI-induced responses in the Transwell migration assay. ANOVA with Bonferroni's *post hoc* test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

LPI content in human plasma. Mass spectrometry data revealed that, in particular, LPI (18:0) was increased in plasma samples of colon cancer patients when compared with healthy controls (Figure 6E), while total LPI (16:0, 18:0, 24:0) only tended to be increased (probably due to the low sample size, ~40% power) (Figure 6F).

Discussion and conclusion

GPCRs activated by lysophospholipid ligands, for example lysophosphatidic acid (LPA) receptors, are regarded as important players in the development of cancer (Dorsam and Gutkind, 2007). This raises the possibility that other lysophospholipid ligand-responsive GPCRs like GPR55 could exert protumorigenic behaviour in a similar way. In parallel, high levels of endogenous ligands of these GPCRs may add to carcinogenesis. For instance, LPI, the endogenous GPR55

ligand, has been reported to be elevated in the blood of patients with ovarian cancer (Shen *et al.*, 2001). The pathophysiological function of the GPR55-LPI axis has, therefore, gained increasing attention as a potential driving force in cancer (Ross, 2011). In our present study, we demonstrated that the GPR55-LPI axis plays an important role in the metastatic behaviour of colon cancer cells *in vitro* and *in vivo*.

One of the main features of metastasis is the adherence of cancer cells to the endothelial lining and the colonization of secondary organs (Hanahan and Weinberg, 2011). By use of the recently characterized selective GPR55 antagonist CID16020046 (Kargl *et al.*, 2013a; Kotsikorou *et al.*, 2013) and through GPR55 siRNA knockdown, we show that adherence of HCT116 colon cancer cells to endothelial cells was significantly inhibited. The GPR55 antagonist also inhibited invasion and migration of the cancer cells in the chemotactic assays. These data clearly indicate a crucial role for GPR55 in colon cancer cell migration and for LPI in the integrity of the endothelial barrier challenged by HCT116 cells. It is not

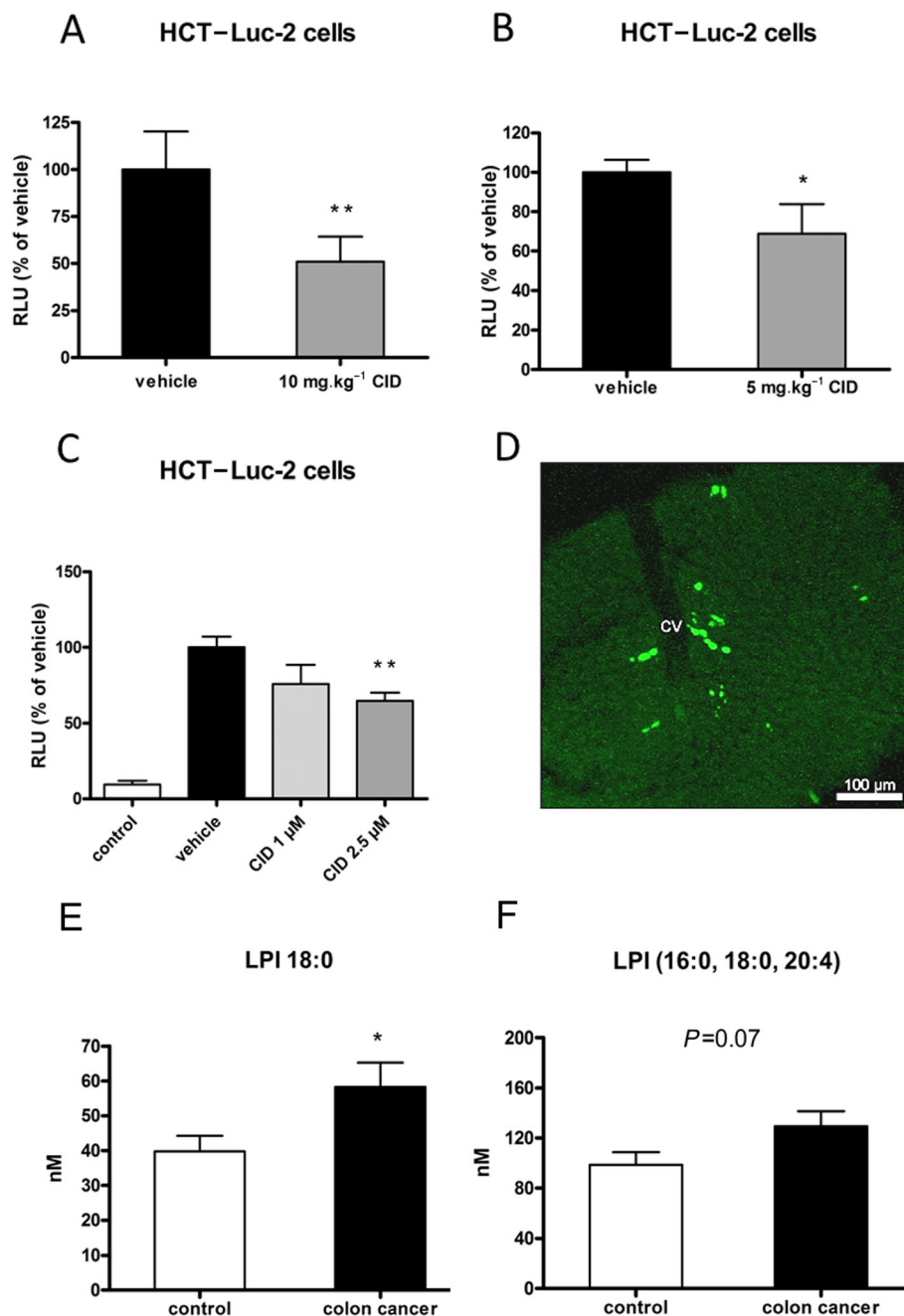


Figure 6

GPR55 promotes cancer colon cell arrest in the liver. (A, B) Pretreatment of C57BL/6 mice with an i.p. injection of 10 mg.kg⁻¹ CID16020046 (CID) and 5 mg.kg⁻¹ cannabidiol (CBD) 30 min before intrasplenic injection of HCT116-CMVp-Luc cells decreased arrest of these cells in the liver. $n = 5-9$; Student's *t*-test. (C) Pretreatment of HCT116-CMVp-Luc cells with CID (1 and 2.5 μM for 30 min) also dose-dependently lowered cell arrest of these cells after intrasplenic application into C57BL/6 mice. $n = 5-10$; ANOVA; Tukey's *post hoc* test. (D) Image shows green fluorescent EGFP transfected HCT116/Luc-2 in a 30 μm thick section of liver tissue 3.5 h after intrasplenic injection of 1×10^6 cells in 50 μL volume into C57BL/6 mice to visually demonstrate arrested cancer cells in the liver. The left liver lobe was frozen on dry ice; sections were cut on a cryostat and mounted on slides. Images were taken under a fluorescent microscope (Olympus IX 70) with a Hamamatsu ORCA CCD camera and xcellence® Olympus imaging software (Olympus, Vienna, Austria). Transient pcDNA-EGFP plasmid transfection was performed using Lipofectamine 2000 following the manufacturer's instructions (Life Technologies). (E, F) The endogenous ligand of GPR55, LPI, was evaluated by LC-MS in blood samples of colon cancer patients and healthy individuals (control); $n = 7$; Student's *t*-test. * $P < 0.05$; ** $P < 0.01$. LPI (18:0) showed significant increases in colon cancer patients in comparison with healthy subjects, while total LPI (16:0, 18:0, 24:0) showed a slight but non-significant increase compared with controls. CV, central vein; RLU, relative light units.

clear, how LPI-treated HCT116 cells alter the endothelial integrity. However, it should be noted that LPI is an inducer of nuclear factor of activated T-cells (NFAT; Henstridge *et al.*, 2009), which has been implicated in cancer cell migration (Mancini and Toker, 2009). One of the genes transactivated by NFAT encodes for autotaxin, which produces LPA, a potent mitogen in breast cancer (rev. in Mancini and Toker, 2009). LPA has been also shown to strongly enhance endothelial permeability (van Nieuw Amerongen *et al.*, 2000), which may have been the case in our experimental set-up. An activated LPI-GPR55 axis is likely present in colon cancer patients who show increased blood levels of LPI as observed in our study. High levels of LPI could easily influence interaction of cancer cells with endothelia because it is not only produced in endothelial and cancer cells (Martin and Wysolmerski, 1987; Piñeiro *et al.*, 2011) but also in neutrophils (Smith and Waite, 1992) and platelets (Billah and Lapetina, 1982), that is, in cells that assist invading tumour cells (Hanahan and Weinberg, 2011).

We also investigated the effects of cannabidiol, a non-psychoactive natural cannabinoid, on adhesion and migration of HCT116 colon cancer cells and whether cannabidiol effects could be mediated by GPR55. It has been previously reported that migration and invasion of cancer cells are inhibited by cannabidiol (Vacani *et al.*, 2005; Ramer *et al.*, 2012). In events like these, cannabidiol could act as an antagonist at GPR55 (Ryberg *et al.*, 2007; Anavi-Goffer *et al.*, 2012; Kotsikourou *et al.*, 2013); however, cannabidiol has been also described as a ligand of other receptors, such as TRPV2, PPAR γ and 5-HT $_{1A}$ receptors (Izzo *et al.*, 2009). In particular, TRPV2, a non-selective cation channel with Ca $^{2+}$ permeability, can be activated by cannabidiol in cancer cells (Nabissi *et al.*, 2013). This could have changed Ca $^{2+}$ homeostasis in the HCT116 cells and influenced migration and adhesion (Prevarskaya *et al.*, 2011). Nevertheless, in our assays, inhibition of adhesion/migration by cannabidiol was clearly diminished by GPR55 siRNA knockdown indicating a major role of the GPR55 receptor in the anti-migratory and anti-adhesion effects of cannabidiol.

Oncogenic properties of GPR55 in cancer cells involve ERK1/2 activity and Ca $^{2+}$ signalling (Andradas *et al.*, 2011; Piñeiro *et al.*, 2011). In a recent work, we were able to show that the GPR55 antagonist CID16020046 inhibited LPI-induced responses in GPR55-HEK293 cells, such as Ca $^{2+}$ flux and ERK1/2 activity (Kargl *et al.*, 2013a). We were, therefore, interested whether the process of adhesion and migration may include these typical GPR55-activated pathways. Blockade of ERK1/2, but not of ROCK, inhibited adhesion and migration of HCT116 cells on endothelial cells indicating that the ERK1/2 pathway may not only be important for GPR55-mediated proliferation of cancer cells (Andradas *et al.*, 2011) but also for cancer cell adhesion to and migration through the endothelium. For all our assays, we used the highly selective MEK1/2 inhibitor PD184161, which is structurally almost identical to PD184352, a MEK inhibitor with no known inhibition of other protein kinases (up to 10 μ M) and a selectivity superior to U0126 (Davies *et al.*, 2000). Interestingly, the ROCK inhibitor Y27632 increased adhesion and migration of HCT116 cells, a finding also observed by others (Wang *et al.*, 2010; Adachi *et al.*, 2011). Another ROCK inhibitor, H-1152, was also ineffective in inhibiting migration and

adhesion suggesting that alternative or additional signalling pathways to Rho/ROCK exist in the migration of colon cancer cells.

To explore the relevance of the GPR55-LPI axis in colon cancer patients, we evaluated LPI content in the blood of colon cancer patients in comparison with healthy individuals. We detected significantly increased LPI (18:0) content in plasma from colon cancer patients possibly indicating an activated LPI-GPR55 axis. In a previous study, stearic (18:0) acid has been shown to constitute one of the major fatty acid components of LPI in serum of healthy individuals and patients with ovarian cancer (Shen *et al.*, 2001), which may be a reason why we measured significant differences only for the LPI (18:0) species and not for total LPI between colon cancer patients and healthy subjects. Piñeiro *et al.* (2011) already demonstrated that LPI activates GPR55 in prostate and ovarian cancer cells through Ca $^{2+}$ mobilization and phosphorylation of Akt and ERK1/2. Because Ca $^{2+}$ mobilization in PC-3 cancer cells by LPI also correlated with increased migration (Monet *et al.*, 2009) and proliferation (Piñeiro *et al.*, 2011), an involvement of LPI in growth and metastasis of colon cancer cells is quite conceivable. LPI was also reported to enhance migration of the metastatic breast cancer cell line MDA-MB-231, which expresses high levels of GPR55 (Ford *et al.*, 2010). In accordance with that study, we could also show that GPR55 overexpression in HCT116 cells enhanced the GPR55 antagonist-sensitive migratory response to LPI. Thus – next to ovarian cancer – colon cancer patients reveal increased LPI levels suggesting LPI as an interesting biomarker candidate for colon cancer.

We finally employed a mouse model and investigated *in vivo* whether GPR55 may alter adhesion and migratory behaviour of colon cancer cells in organs that are first colonized by colon tumour cells. Treatment of mice with the GPR55 inhibitor or cannabidiol diminished the arrest of HCT116-CMVp-Luc cells in the liver as evaluated by luciferase activity. The GPR55 antagonists, therefore, slowed down metastasis of liver tissue with colon cancer cells. When only HCT116-CMVp-Luc cells were pretreated with CID16020046 before injection, this had the same inhibitory effect, which proved that GPR55 expressed on the cancer cells rather than endothelial cells were mediating the metastatic effect.

To summarize, in the present study, we demonstrated that GPR55 is involved in metastatic behaviour of colon cancer cells such as adhesion, invasion and migration and in the arrest of colon cancer cells in liver tissue. These properties were blocked by GPR55 antagonism. Together with the increase of LPI levels in colon cancer patients and the presence of GPR55 in colon tumour cells, it is suggested that the LPI-GPR55 axis could be involved in the metastasis of colon cancer cells. In light of the limited therapeutic options to interfere with metastasis of colon cancer, further studies are needed to reveal insight into GPR55-mediated mechanisms of metastasis.

Acknowledgements

We would like to thank Veronika Pommer for excellent technical assistance and Dr Jens Hoffmann and Dr Antje Siegert from EPO for the HCT116-CMVp-Luc cells. This study was supported by Austrian Science Fund (FWF grants P25633

and P22771 to R. S. and P22521-B18 to A. H.), the Austrian National Bank (OeNB 14429 to R. S. and 14263 to A. H.) and the Innovative Medicines Initiative Joint Undertaking (IMI) grant (grant agreement no. 115234, OncoTrack to J. H.). J. K. is funded by the PhD and the START Funding programme of the Medical University of Graz.

Author contributions

R S, J K, J H and A H designed the study and supervised the research. J K, L A, C H, D F and A S conducted *in vitro* assays and analysed the results. A F and C M carried out the LC-MS studies. R S performed the *in vivo* experiments. J H, A E-H, S L and S U performed sample collection and analysis of the patients' material. R S, J K, L A, J H, A F and A H wrote the manuscript. All authors were involved in data discussion and critical reviewing of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

References

- Adachi S, Yasuda I, Nakashima M, Yamauchi T, Yoshioka T, Okano Y, *et al.* (2011). Rho-kinase inhibitor upregulates migration by altering focal adhesion formation via the Akt pathway in colon cancer cells. *Eur J Pharmacol* 650: 145–150.
- Alexander SP, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Catterall WA, *et al.* (2013a). The Concise Guide to PHARMACOLOGY 2013/14: ion channels. *Br J Pharmacol* 170: 1459–1581.
- Alexander SP, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, *et al.* (2013b). The Concise Guide to PHARMACOLOGY 2013/14: G protein-coupled receptors. *Br J Pharmacol* 170: 1607–1651.
- Alexander SP, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, *et al.* (2013c). The Concise Guide to PHARMACOLOGY 2013/14: nuclear hormone receptors. *Br J Pharmacol* 170: 1652–1675.
- Alexander SP, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, *et al.* (2013d). The Concise Guide to PHARMACOLOGY 2013/14: enzymes. *Br J Pharmacol* 170: 1797–1867.
- Anavi-Goffer S, Baillie G, Irving AJ, Gertsch J, Greig IR, Pertwee RG, *et al.* (2012). Modulation of L- α -lysophosphatidylinositol/GPR55 mitogen-activated protein kinase (MAPK) signaling by cannabinoids. *J Biol Chem* 287: 91–104.
- Andradas C, Caffarel MM, Pérez-Gómez E, Salazar M, Lorente M, Velasco G, *et al.* (2011). The orphan G protein-coupled receptor GPR55 promotes cancer cell proliferation via ERK. *Oncogene* 30: 245–252.
- Aviello G, Romano B, Borrelli F, Capasso R, Gallo L, Piscitelli F, *et al.* (2012). Chemopreventive effect of the non-psychoactive phytocannabinoid cannabidiol on experimental colon cancer. *J Mol Med (Berl)* 90: 925–934.
- Balenga NA, Aflaki E, Kargl J, Platzer W, Schröder R, Blättermann S, *et al.* (2011). GPR55 regulates cannabinoid 2 receptor-mediated responses in human neutrophils. *Cell Res* 21: 1452–1469.
- Billah MM, Lapetina EG (1982). Formation of lysophosphatidylinositol in platelets stimulated with thrombin or ionophore A23187. *J Biol Chem* 257: 5196–5200.
- Davies SP, Reddy H, Caivano M, Cohen P (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 351: 95–105.
- Dorsam RT, Gutkind JS (2007). G-protein-coupled receptors and cancer. *Nat Rev Cancer* 7: 79–94.
- Fauland A, Köfeler H, Trötz Müller M, Knopf A, Hartler J, Eberl A, *et al.* (2011). A comprehensive method for lipid profiling by liquid chromatography-ion cyclotron resonance mass spectrometry. *J Lipid Res* 52: 2314–2322.
- Ford LA, Roelofs AJ, Anavi-Goffer S, Mowat L, Simpson DG, Irving AJ, *et al.* (2010). A role for L- α -lysophosphatidylinositol and GPR55 in the modulation of migration, orientation and polarization of human breast cancer cells. *Br J Pharmacol* 160: 762–771.
- Fredriksson R, Lagerström MC, Lundin LG, Schiöth HB (2003). The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol* 63: 1256–1272.
- Gladbach A, van Eersel J, Bi M, Ke YD, Ittner LM (2014). ERK inhibition with PD184161 mitigates brain damage in a mouse model of stroke. *J Neural Transm* 121: 543–547.
- Hanahan D, Weinberg RA (2011). Hallmarks of cancer: the next generation. *Cell* 144: 646–674.
- Henstridge CM, Balenga NA, Ford LA, Ross RA, Waldhoer M, Irving AJ (2009). The GPR55 ligand L- α -lysophosphatidylinositol promotes RhoA-dependent Ca^{2+} signaling and NFAT activation. *FASEB J* 23: 183–193.
- Henstridge CM, Balenga NA, Kargl J, Andradas C, Brown AJ, Irving A, *et al.* (2011). Minireview: recent developments in the physiology and pathology of the lysophosphatidylinositol-sensitive receptor GPR55. *Mol Endocrinol* 25: 1835–1848.
- Huang L, Ramirez JC, Frampton GA, Golden LE, Quinn MA, Pae HY, *et al.* (2011). Anandamide exerts its antiproliferative actions on cholangiocarcinoma by activation of the GPR55 receptor. *Lab Invest* 91: 1007–1017.
- Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R (2009). Non-psychoactive plant cannabinoids: new therapeutic opportunities from an ancient herb. *Trends Pharmacol Sci* 30: 515–527.
- Juneja J, Casey PJ (2009). Role of G12 proteins in oncogenesis and metastasis. *Br J Pharmacol* 158: 32–40.
- Kargl J, Balenga N, Parzmair GP, Brown AJ, Heinemann A, Waldhoer M (2012). The cannabinoid receptor CB₁ modulates the signaling properties of the lysophosphatidylinositol receptor GPR55. *J Biol Chem* 287: 44234–44248.
- Kargl J, Brown AJ, Andersen L, Dorn G, Schicho R, Waldhoer M, *et al.* (2013a). A selective antagonist reveals a potential role of G protein-coupled receptor 55 in platelet and endothelial cell function. *J Pharmacol Exp Ther* 346: 54–66.
- Kargl J, Haybaeck J, Stančić A, Andersen L, Marsche G, Heinemann A, *et al.* (2013b). O-1602, an atypical cannabinoid, inhibits tumor growth in colitis-associated colon cancer through multiple mechanisms. *J Mol Med (Berl)* 91: 449–458.
- Kotsikourou E, Sharir H, Shore DM, Hurst DP, Lynch DL, Madrigal KE, *et al.* (2013). Identification of the GPR55 antagonist binding site using a novel set of high-potency GPR55 selective ligands. *Biochemistry* 52: 9456–9469.
- Lauckner JE, Jensen JB, Chen HY, Lu HC, Hille B, Mackie K (2008). GPR55 is a cannabinoid receptor that increases intracellular calcium and inhibits M current. *Proc Natl Acad Sci U S A* 105: 2699–2704.

- Mancini M, Toker A (2009). NEAT proteins: emerging roles in cancer progression. *Nat Rev Cancer* 9: 810–820.
- Martin TW, Wysolmerski RB (1987). Ca^{2+} -dependent and Ca^{2+} -independent pathways for release of arachidonic acid from phosphatidylinositol in endothelial cells. *J Biol Chem* 262: 13086–13092.
- Matyash V, Liebisch G, Kurzchalia TV, Shevchenko A, Schwudke D (2008). Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. *J Lipid Res* 49: 1137–1146.
- McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. *Br J Pharmacol* 160: 1573–1576.
- Monet M, Gkika D, Lehen'kyi V, Pourtier A, Vanden Abeele F, Bidaux G, *et al.* (2009). Lysophospholipids stimulate prostate cancer cell migration via TRPV2 channel activation. *Biochim Biophys Acta* 1793: 528–539.
- Nabissi M, Morelli MB, Santoni M, Santoni G (2013). Triggering of the TRPV2 channel by cannabidiol sensitizes glioblastoma cells to cytotoxic chemotherapeutic agents. *Carcinogenesis* 34: 48–57.
- Oka S, Toshida T, Maruyama K, Nakajima K, Yamashita A, Sugiura T (2009). 2-Arachidonoyl-sn-glycero-3-phosphoinositol: a possible natural ligand for GPR55. *J Biochem* 145: 13–20.
- Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP, *et al.* (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledgebase of drug targets and their ligands. *Nucl Acids Res* 42 (Database Issue): D1098–106.
- Pérez-Gómez E, Andradas C, Flores JM, Quintanilla M, Paramio JM, Guzmán M, *et al.* (2012). The orphan receptor GPR55 drives skin carcinogenesis and is upregulated in human squamous cell carcinomas. *Oncogene* 32: 2534–2542.
- Piñeiro R, Maffucci T, Falasca M (2011). The putative cannabinoid receptor GPR55 defines a novel autocrine loop in cancer cell proliferation. *Oncogene* 30: 142–152.
- Prevarskaia N, Skryma R, Shuba Y (2011). Calcium in tumour metastasis: new roles for known actors. *Nat Rev Cancer* 11: 609–618.
- Rajput A, Dominguez San Martin I, Rose R, Beko A, Levea C, Sharratt E, *et al.* (2008). Characterization of HCT116 human colon cancer cells in an orthotopic model. *J Surg Res* 147: 276–281.
- Ramer R, Bublitz K, Freimuth N, Merkord J, Rohde H, Hausteil M, *et al.* (2012). Cannabidiol inhibits lung cancer cell invasion and metastasis via intercellular adhesion molecule-1. *FASEB J* 26: 1535–1548.
- Ross RA (2011). L- α -lysophosphatidylinositol meets GPR55: a deadly relationship. *Trends Pharmacol Sci* 32: 265–269.
- Ryberg E, Larsson N, Sjögren S, Hjorth S, Hermansson NO, Leonova J, *et al.* (2007). The orphan receptor GPR55 is a novel cannabinoid receptor. *Br J Pharmacol* 152: 1092–1101.
- Sack U, Wölther W, Scudiero D, Selby M, Kobelt D, Lemm M, *et al.* (2011). Novel effect of antihelminthic Niclosamide on S100A4-mediated metastatic progression in colon cancer. *J Natl Cancer Inst* 103: 1018–1036.
- Shen Z, Wu M, Elson P, Kennedy AW, Belinson J, Casey G, *et al.* (2001). Fatty acid composition of lysophosphatidic acid and lysophosphatidylinositol in plasma from patients with ovarian cancer and other gynecological diseases. *Gynecol Oncol* 83: 25–30.
- Smith DM, Waite M (1992). Phosphatidylinositol hydrolysis by phospholipase A2 and C activities in human peripheral blood neutrophils. *J Leukoc Biol* 52: 670–678.
- Staton PC, Hatcher JP, Walker DJ, Morrison AD, Shapland EM, Hughes JP, *et al.* (2008). The putative cannabinoid receptor GPR55 plays a role in mechanical hyperalgesia associated with inflammatory and neuropathic pain. *Pain* 139: 225–236.
- Vaccani A, Massi P, Colombo A, Rubino T, Parolaro D (2005). Cannabidiol inhibits human glioma cell migration through a cannabinoid receptor-independent mechanism. *Br J Pharmacol* 144: 1032–1036.
- van Nieuw Amerongen GP, Vermeer MA, van Hinsbergh VW (2000). Role of RhoA and Rho kinase in lysophosphatidic acid-induced endothelial barrier dysfunction. *Arterioscler Thromb Vasc Biol* 20: E127–E133.
- Wang L, Xue L, Yan H, Li J, Lu Y (2010). Effects of ROCK inhibitor, Y-27632, on adhesion and mobility in esophageal squamous cell cancer cells. *Mol Biol Rep* 37: 1971–1977.
- Wiggins H, Rappoport J (2010). An agarose spot assay for chemotactic invasion. *Biotechniques* 48: 121–124.
- Zhang X, Maor Y, Wang JF, Kunos G, Groopman JE (2010). Endocannabinoid-like N-arachidonoyl serine is a novel pro-angiogenic mediator. *Br J Pharmacol* 160: 1583–1594.

Supporting Information

Additional Supporting Information may be found in the on-line version of this article at the publisher's web-site:
<http://dx.doi.org/10.1111/bph.13345>

Figure S1 HCT116 colon cancer cells express GPR55 mRNA. GPR55 expression was evaluated in several colon cancer cell lines (HT29, SW480, HCT116; all from Interlab Cell Line Collection, Genoa, Italy). Compared to two other colon cancer cell lines (SW480 and HT29), qPCR showed that HCT116 expressed the highest copy numbers of GPR55. We therefore decided to continue with HCT116 cells for all the following experiments.

Figure S2 Viability of HCT116 cells was measured after 17 hrs upon treatment with increasing concentrations of cannabidiol (CBD), GPR55 antagonist CID16020046 (CID) and PD184161 using the MTS assay (Cell Titer 96® AQueous One Solution Cell Proliferation Assay). Absorbance was measured at 490 nm after incubating the cells with MTS substrate for 60–100 min and afterwards normalized to vehicle. At the indicated concentrations, CID and cannabidiol did not influence cell viability. Higher concentrations of PD184161 (10 μM) significantly reduced cell viability. Data are means \pm SEM from at least 4 independent experiments performed in triplicates. ANOVA; Tukey's post-hoc test.

Figure S3 (A) Incubation with ROCK inhibitor H-1152 (10 nM) had no significant effect on the migration of LPI induced migratory responses of GPR55 overexpressing HCT116 in the Transwell migration assay (*veh*, vehicle = DMSO; $n = 6$ –8; one-way ANOVA; Tukey's post hoc test). (B) Incubation with Rock inhibitor H-1152 (10 nM) had no effect on the adhesion of naïve HCT116 cells onto a HUVEC cell monolayer ($n = 6$; t-test; *n.s.*, not significant).

Figure S4 PCR amplification of GPR55 transcripts. Gel showing bands of amplicons of passages 4 (p4) and 7 (p7) from HCT116 cancer cells and passage 6 (p6) of HCT116-CMVp-Luc cancer cells (HCT116-Luc). HCT116 as well as HCT116-CMVp-Luc cancer cells express GPR55 transcripts. Amplicons were electrophoresed in 1% agarose gel and stained with ethidiumbromide. GPR55 pcDNA3.1 plasmid (10 ng; Kargl *et al.*, 2012) was used as positive control.